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Toxicity of neem seed extract to *Tessaratoma papillosa* (Drury) relative to its allozyme genotypes

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Abstract: The relationships between the susceptibility to neem seed extract and the allozyme genotypes were examined in the first instar nymphs of *Tessaratoma papillosa* (Drury) for two polymorphic enzyme loci of *Pgi* and *Mdh* using allozyme analysis. Acute exposures of the insect to 5.2 mg/mL (LC_{50} value) neem seed extract resulted in 51.8% mortality in 24 h. Under the given experimental conditions, insect mortalities were significantly different among certain genotypes and alleles. At locus *Pgi*, the insects with the *Pgi-bb* genotype displayed the highest mortality (84%), whereas those with *Pgi-aa* and *Pgi-cc* showed the lowest mortalities (0 and 7%, respectively), which were significantly different from that of *Pgi-bb*. At locus *Mdh*, the insects with the genotype *Mdh-cc* and *Mdh-aa* exhibited the highest mortality (93%), but no mortality was observed in the insects with the genotype *Mdh-cc*. These results clearly indicated that the insects with genotype *Mdh-aa* and *Mdh-cc* were significantly different from those with other three genotypes *Mdh-ab*, *Mdh-bb* and *Mdh-bc* in response to neem seed extract. In contrast, the mortalities of the insects with the *Pgi-a* and *Mdh-c* allele were the lowest, and were significantly different from those with other alleles. Our studies showed that individuals of *T. papillosa* with different genotypes had significantly different responses to neem seed extract. Such distinct relationships between the insect susceptibility to neem seed extract and its allozyme genotypes may allow us to use certain genotypes and alleles as genetic markers to assess the susceptibility of *T. papillosa* to neem seed extract.

Key words: *Tessaratoma papillosa*; neem seed extract; allozymes; genotype; allele; selective lethal effect

1 INTRODUCTION

Tessaratoma papillosa (Drury) is an impotant pest of lichi and longan. It mainly damages litchi, longan, and citrus fruit trees, and may cause great economic loss by decreasing the output and quality of the fruits if not properly prevented and cured. The bug sucks juices of tender tips, spica and young fruits of the trees by stinging them with its needle-shaped mouthpart, and causes the shedding of flower and fruits (Xie *et al.*, 2004). Being frightened, it shoots foul-smelling liquid that can burn or darken the flowers, tender tips, tender leaves and young fruits, and even lead to downy mildew (Xu *et al.*, 1993). For a long time, controlling of *T. papillosa* mainly depends on using synthetic insecticides. Although beneficial, extensive use of these chemicals has created public concern about their effects on the environment and on human health. Consequently, intensive efforts are being made to find alternatives, especially insecticides of plant origin, which are readily biogradable, and perceived to be environmentally safe and ecologically acceptable.

Neem extract is one kind of the best bio-pesticides and is used widely to control insect pests. Neem-based insecticides containing azadirachtin have been reportedly used to control over 400 species of insects belonging to the orders Diptera, Hymenoptera, Coleoptera, Lepidoptera, Orthoptera and Hemiptera, including important agricultural pests, such as armyworms, leafminers, aphids, and whiteflies (Schmutterer, 1990; Uleichs *et al.*, 2001; Haseeb *et al.*, 2004; Terezinha *et al.*, 2004; Shoil *et al.*, 2005). Neem-based insecticides should be ideal alternatives to synthetic insecticides for controlling fruit tree, vegetable and tea insect pests. Since it is selective, neem presents a less negative impact on the ecosystems, and its association with biological control has been paid to more and more attention (Terezinha *et al.*, 2004). However to our knowledge, neem-based insecticides have not been used in the control of *T. papillosa*. Could it be used to control *T. papillosa*? How effective is it in controlling *T. papillosa*? With these questions in mind, we tested the toxicity of neem seed extract to *T. papillosa*.

Many literatures focused on the lethal relationship between environmental contamination and aquatic organisms via allozyme analysis, and evaluated the

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effects of contamination on a population's genetic structure (Guttman, 1994; Fore *et al*., 1995; Duan *et al*., 2000a, 2000b, 2001). The results indicated that the genetic distance analysis could be a more sensitive tool in demonstrating the overall genetic disturbance caused by environmental change and potential use of genetic distances in these organisms as a bioindicator for monitoring environmental changes (Duan *et al*., 2000a, 2000b, 2001). It had been used to resolve questions such as the identification of resistant and sensitive genotypes, the relationship between genetic diversity and tolerance, and how genotype and/or allele frequencies change after exposure to contaminants (Guttman, 1994). As an excellent reference, the theory and method had been adopted to study the correlation between insecticide toxicity and the allozyme genotypes and/or alleles of *Oxya chinensis* and *Plutella xylostella* (Li and Qiao, 2000; Li *et al*., 2004a, 2004b, 2004c; Lu *et al*., 2004a, 2004b). Researches have provided a new idea that the dynamic variability of allozyme genotypes and/or alleles can be taken as a potential genetic indicator to monitor pests' resistance to insecticides.

In this study, the toxicity of neem seed extract to the first instar nymphs of *T. papillosa* was tested, so that effects of neem-based insecticides on *T. papillosa* can be preliminarily evaluated. The allozyme analysis was used to assess the resistant risk of neem-based insecticides to *T. papillosa* by determining whether the insect's survival was associated with specific genotypes at selected enzyme loci during exposure. Malate dehydrogenase (MDH, E.C. 1.1.1.37) and glucose-6-phosphate isomerase (PGI, E.C. 5.3.1.9) were chosen because of their polymorphism and high activity in *T. papillosa* under our experiment conditions, and were extensively studied in allozyme analysis (Li *et al*., 2004a, 2004b, 2004c; Lu *et al*., 2004a, 2004b).

2 MATERIALS AND METHODS

2.1 Sample collection

The first instar nymphs of *T. papillosa* were collected from the research base of Chinese Academy of Tropical Agricultural Sciences in Danzhou, Hainan, and fed with fresh litchi leaves in insect net at room temperature before conducting acute toxicity treatment with neem seed extract.

2.2 Insecticide bioassays

The neem seed extract was provided by Laboratory of Insect Toxicology, South China Agricultural University.

Susceptibility of the first instar nymphs of *T. papillosa* to neem seed extract was evaluated using topical application. Five different concentrations (0.001, 0.003, 0.007, 0.01 and 0.03 g/mL) of neem seed extract were prepared with ethanol as the solvent. The bioassay was carried out with five neem seed extract doses as treatments and a solvent as the control. Each treatment or control was repeated three times, and there were twenty individuals in each replicate. The insects were then put in Petri dishes with fresh litchi leaves at room temperature. Mortality was determined after 24 h.

2.3 Acute toxicity treatment

With the same topical application, a sample of first instar nymphs of *T. papillosa* was treated with the concentration of LC_{50} neem seed extract based on the result of 2.2. After 24 h, dead and surviving individuals were stored at −80℃ individually before electrophoresis.

2.4 Allozyme electrophoresis

Allozyme analysis was performed using horizontal starch gel electrophoresis (Richardsom, 1986; Wang, 1998; Li *et al*., 2004). The 12.0% (w/v) gel was prepared using a mixture of soluble starch and potato starch (Sigma) at a ratio of 1∶1 for electrophoresis. Phosphate buffer (0.05 moL/L, pH 8.0) was used as electrode buffer, and the ratio of electrode buffer to gel buffer was 9∶1.

The leg muscle of each insect was removed and homogenized in 20 μL double distilled water on an ice pan.

The sample loading and gel staining were carried according to Murphy *et al*. (1996) and Wang (1998). Gels were run at constant voltage of 290 V for about 4 and a half hours with crushed ice as coolant at the top of gel at 4℃ in a refrigerator.

Two polymorphic enzymes were examined: malate dehydrogenase (MDH, E.C. 1.1.1.37) and glucose-6-phosphate isomerase (PGI, E.C. 5.3.1.9). Alleles were identified by labeling the fastest migrating allele with *a*, the second fastest allele with *b*, and so on.

2.5 Data analysis

Concentration-mortality regression lines were maintained with SAS. The differential mortality among the allozyme genotypes and alleles of treated *T. papillosa* were compared by contingency table χ^2 test, herein, each genotype as a group and the individual number as replicate.

The genetic structures of the two allozyme genotypes were determined via BIOSYS-2 (Swofford *et al*., 1981), with which allele frequency, percent polymorphic loci, heterozygosity (H), fixation index (F), goodness-of-fit to Hardy-Weinberg's (H-W) equilibrium and Roger's genetic distance (D) were calculated.

3 RESULTS

3.1 The toxicity determination

The mortality of the first instar nymphs of *T.*

papillosa exposed to neem seed extract depended upon the concentration of extract and a linear relationship was determind. The linear relationship was $y = 1.54 + 0.456x$, LC_{50} was 5.2 mg/mL and R^2 was 0.7804. The mortality of the first instar nymphs of *T. papillosa* at the lowest and the highest concentration was 16.67% and 88.33%, respectively.

3.2 The correlation analysis between mortality and genotypes

The mean mortality of 193 first instar nymphs of *T. papillosa* at the concentration of 5.2 mg/mL was 51.80%.

Overall, different lethal effect was observed at two different loci of different genotypes (Table 1). At the locus *Pgi*, significant differences were observed among the genotypes of high, medium and low mortality individuals. Individuals with *Pgi-bc* experienced the highest mortality (84%), but all individuals with *Pgi-aa* genotype survived. At the locus *Mdh*, *Mdh-aa* experienced the highest mortality, but all individuals with *Mdh-cc* genotype survived.

Table 1 Genotype effects on the probability of the two polymorphic loci *Pgi* and *Mdh* in *T. papillosa* population treated with neem seed extract

Locus	Mortality (%)				
	aa	*ab*	*bb*	*bc*	*cc*
Pgi	0 (29) a	58 (40) b	64 (61) b	84 (44) c	7 (15) a
Mdh	93 (15) a	54 (28) b	52 (96) b	61 (51) b	0 (13) c

Notes: The numbers in parenthesis were the total individual numbers including surviving and dead individuals with corresponding genotype. The data within a row followed with different letters were significantly different at 0.05 level. The same for Table 2.

3.3 The correlation analysis between mortality and alleles

The lethal effects of neem seed extract to *T. papillosa* were also different at the level of alleles (Table 2). χ^2 tests showed significant difference among the three alleles of *Pgi*. Allele *Pgi-b* that occurred in the most individuals exhibited the highest mortality, *Pgi-a* exhibited the lowest mortality, while the mortality of *Pgi-c* was medium. At *Mdh* locus, the mortalities of allele *Mdh-a* and *Mdh-b* showed no difference ($P < 0.05$), but both showed a significant different lethal effect when compared with *Mdh-c* allele. However, the proportion of individuals with high mortality alleles was the highest of at least 75% at *Pgi* locus and 80% at *Mdh* locus.

Table 2 χ^2 tests for the mortality difference between *Pgi* and *Mdh* alleles of surviving and dead group of *T. papillosa* treated with neem seed extract

Locus	Mortality (%)		
	a	*b*	*c*
Pgi	23 (98) a	70 (226) b	52 (74) c
Mdh	61 (38) a	54 (271) a	40 (77) b

3.4 The allozyme genetic background analysis

When using the buffer system, the two enzymes all migrated from cathode to anode and each was found to have three allele polymorphic loci. At the two loci, the most frequent allele was the same, *Pgi-b* and *Mdh-b* (Table 3). The genotype frequencies at the two loci were all deviated significantly from Hardy-Weinberg equilibrium.

The frequency of the allele "*b*" had been elevated in the dead group in comparison with that of the initial group. The heterozygotes in dead groups were some exceeding since the *F* values in the two enzymes was lower than zero and the H_o was higher than H_e, but that was just opposite in alive and initial groups (Table 3, 4).

The genetic identity (*I*) was higher between the alive and initial group as well as between the dead and initial group (Table 5) than that between the alive and dead group. The genetic distance (*D*) was greater between the alive and dead group than that between the alive and initial group as well as between the dead and initial group.

Table 3 Allele frequency and tests for Hardy-Weinberg (H-W) expectations at *Pgi* and *Mdh* loci of *T. papillosa* treated with neem seed extract

Alleles	*Pgi*			*Mdh*		
	Alive	Dead	Initial	Alive	Dead	Initial
Sample size	93	100	193	93	100	193
a	0.425	0.115	0.264	0.081	0.115	0.098
b	0.366	0.690	0.534	0.672	0.730	0.702
c	0.210	0.195	0.202	0.247	0.155	0.199
χ^2	60.070**	16.073**	43.030**	19.025**	15.153**	17.212**
F	0.531	−0.269	0.246	0.262	−0.070	0.106

Initial: including survival and dead. ** $P < 0.01$; *F*: Fixation index.

Table 4 Genetic variability at the two loci in each *T. papillosa* group treated with neem seed extract

Groups	Mean sample size per locus	Mean number of alleles per locus	Percentage of loci polymorphic*	Mean heterozygosity	
				H_o	H_e**
Alive	93.0 (0.0)	3.0 (0.0)	100	0.328 (0.027)	0.564 (0.081)
Dead	100.0 (0.0)	3.0 (0.0)	100	0.530 (0.070)	0.454 (0.022)
Initial	193.0 (0.0)	3.0 (0.0)	100	0.433 (0.023)	0.532 (0.074)

H_o: observed heterozygosity. H_e: Hardy-Weinberg expected heterozygosity. * A locus is considered polymorphic if the most common allele does not exceed 0.95 (standard errors in-parentheses); ** Unbiased estimate (see Nei, 1978).

Table 5 Nei (1978) unbiased genetic identity (I: below diagonal) and modified Roger's genetic distance (D: above diagonal) among the three groups of *T. papillosa* treated with neem seed extract

Group	Alive	Dead	Initial
Alive	–	0.232	0.120
Dead	0.902	–	0.112
Initial	0.974	0.982	–

4 DISCUSSION

Data indicate that although the neem products contributed towards pest reduction, but lost its efficacy after treatment and pest population there after increased (Akbar *et al.*, 2003). It has been found that application of neem-based insecticide at recommended application rates do not harm aquatic invertebrates categorized as planktonic and filter feeding. The benthic invertebrate *Chironomus riparius* was, however, affected by multiple applications of neem. High concentrations of neem were possibly not economical once the resistance occurred (Awad, 2003). So, in the experiment, we also did resistance risking evaluation of *T. papillosa* to neem-based insecticide before it could be used to control the insect.

The results of the toxicity of neem seed extract suggested that the neem-based insecticide had efficacy on controlling *T. papillosa*, though such effect was not very well, because the linear slope was not sharp. Then we tested the tolerance of the insect to neem seed extract via correlation analysis between allozyme genotypes of *T. papillosa* and toxic effects of neem seed extract. We hope that an inspecting index and evaluating basis of *T. papillosa* resistance to neem-based insecticide can be provided.

The significant toxin-genotype/allele interaction suggested a differential tolerance of *T. papillosa* to neem seed extract among genotypes and alleles. *Pgi-bc*/*Pgi-aa* genotype was sensitive/tolerant to neem extract, and *Pgi-a* allele was tolerant to the toxicant. It was very interesting that such response of *T. papillosa* to neem seed extract was similar to that of *Oxya chinensis* to avermectin (Li *et al.*, 2004c). In addition to *Pgi*, *Mdh-aa* was sensitive to neem seed extract, but *Mdh-cc* and *Mdh-c* was the highest tolerant genotype and alleles. It demonstrated the correlation between allozyme genotypes and/or alleles mortality of *T. papillosa* and toxic effects of neem seed extract. The neem seed extract had a selective lethal effect on the studied *T. papillosa* population (Table 1, 2).

Insect resistance to pesticides is an evolutionary event, possibly forming the genetic structure alternations within a population under the constant directional selection by pesticides (Tang and Wu, 2000). Identifying the physiological mechanisms of resistance is the first step in the characterization of resistance genes and also a prerequisite for understanding the evolution of insecticide resistance and for resistance management (Tsagkarakou *et al.*, 2002). In the present study, the genotypes and alleles of the two enzyme PGI and MDH might not directly explain the resistance occurrence of *T. papillosa* to neem-based insecticides. But the correlation between the allozyme genotype and the toxic effects of neem seed extract had been demonstrated. Individuals with *Pgi-aa*, *Pgi-cc* and *Mdh-cc* genotypes had the highest tolerance to neem seed extract, and the alleles of *Pgi-a* and *Mdh-c* were the highest tolerant alleles to neem seed extract. It suggested that the increased these genotypes and alleles frequencies in the population will be useful as the potential resistant genetic marker of *T. papillosa* to neem-based insecticide.

In conclusion, to some extent, the neem seed extract had differentiation effect on the genetic structure of *T. papillosa* studied, but the effect was not significant as indicated by the high genetic identity and the low genetic distance between the alive and dead group (Table 5). Thus this may suggest that neem-based insecticides is not likely to cause resistance. The existence of homozygotic individuals in the *T. papillosa* population may be of benefit for them to survive the toxicity of neem-based insecticides. The increased frequency of the most frequent allele in the dead group in comparison with that of the alive and initial group may be a indirect indication for that the neem-based insecticide is an effective pesticide for control of *T. papillosa*.

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印楝种子提取物对荔枝蝽的毒性及与其等位酶基因型之间的关系

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摘要:用 5.2 mg/mL(LC_{50})的印楝种子提取物对荔枝蝽 1 龄若虫进行急性毒性处理,24 h 死亡率为 51.8%。通过等位酶分析检测了死亡与存活试虫两种酶(PGI 和 MDH),两个基因座(*Pgi* 和 *Mdh*)上各基因型及等位基因与印楝种子提取物毒性之间的关系,进行致死性差异比较研究。结果表明,印楝种子提取物对具有不同基因型及等位基因个体的致死性存在差异。在 *Pgi* 基因座上,*Pgi-bb* 基因型死亡率最高,为 84%,*Pgi-aa* 和 *Pgi-cc* 基因型死亡率较低,分别为 0 和 7%,且与死亡率最高的 *Pgi-bb* 基因型存在显著差异($P < 0.05$)。在基因座 *Mdh* 上,*Mdh-aa* 基因型个体死亡率最高(93%),而具有 *Mdh-cc* 基因型的个体全部存活了下来,另外 3 个基因型 *Mdh-ab*、*Mdh-bb* 与 *Mdh-bc* 死亡率居中,都与 *Mdh-aa*、*Mdh-cc* 基因型死亡率之间存在显著差异。在等位基因上,*Pgi-a* 和 *Mdh-c* 个体的死亡率都最低,与各自其他两个等位基因的死亡率之间存在显著差异。结果说明不同基因型个体对印楝提取物具有不同的反应,印楝种子提取物对荔枝蝽等位酶基因型及等位基因存在选择性致死作用。这种荔枝蝽对印楝种子提取物的敏感性与其等位酶基因型及等位基因之间显明的相关关系提示我们,可将荔枝蝽种群中对印楝种子提取物敏感性低的基因型及等位基因作为遗传标记去监测荔枝蝽对印楝种子提取物的抗性状况。

关键词:荔枝蝽;印楝种子提取物;等位酶;基因型;等位基因;选择性致死作用

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